INTERACTION OF RICIN-SENSITIVE AND RICIN-RESISTANT CELL LINES WITH OTHER CARBOHYDRATE-BINDING TOXINS

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1. Introduction

The toxic plant lectin, ricin, consists of 2 polypeptide chains linked by a disulphide bond. One polypeptide subunit binds to cell surface glycoproteins carrying terminal galactose residues following which the toxin enters the cell where the other subunit after dissociation inactivates catalytically 60 S ribosomes causing cell death [1]. Binding to cell surface receptors is an essential first step in ricin toxicity and cell variants selected for growth in the presence of ricin show specific defects in glycoprotein N-glycan assembly leading to a decrease in complex chains containing galactose terminal sequences [2].

Here, we report on the sensitivity of ricin-resistant cell lines to the cytotoxicity of a newly described [3] lectin of the common mistletoe *Viscum album* and modeccin, the toxic principle of *Adenia digitata,* an African plant [4,5]. These lectins, like ricin, recognise galactose residues of glycoproteins. We have also studied the effects of diphtheria toxin on these cells. Diphtheria toxin is believed to bind to cell surface glycoproteins [6] possibly carrying N-glycans of the oligomannosidic type, the synthesis of which is hardly affected in mutants selected for resistance to ricin.

We show that cell lines derived from different species and tissue origins differ markedly in their sensitivity to the galactose-binding lectins.

2. Materials and methods

Baby hamster kidney (BHK C13) cells, ricin-resistant variants Ric^R14, 19 and 21 [7] and mouse L-fibroblasts are laboratory stocks. The cells were cultured at 33 or 37°C as monolayers in Falcon tissue culture-ware in Eagle's medium supplemented with 10% (v/v) foetal calf serum and gentamicin (50 μg/ml). The rat hepatocyte cell line BLSL [8] was kindly provided by R. F. Legg (Toxicology Unit, MRC Laboratories, Carshalton). The cells were grown at 37°C in monolayer culture with Williams medium E (Flow Labs, Irvine), supplemented with 5% foetal calf serum, 2 mM glutamine and gentamicin (50 μg/ml).

Ricin was obtained from Miles Labs; modeccin [4,5] and diphtheria toxin were kindly provided by S. Olsnes (Norsk Hydro's Institute for Cancer Research, Oslo); *Viscum album* L (mistletoe) toxin [3] was kindly provided by R. F. Legg and F. Stirpe (Instituto di Patologia generale dell'Universita di Bologna). The toxins were kept at 5 mg/ml in phosphate-buffered saline (PBS) at −20°C.

The effects of the toxins on cellular protein synthesis were measured using monolayer cultures in Limbro 24 well tissue culture plates. Confluent cultures were rinsed with PBS and incubated with various concentrations of each toxin at 37°C for 3 h except for *Viscum album* lectin which was left with the cells for 18 h at 37°C. The cells were then incubated at 37°C for 1 h with 1–2 μCi/ml of L-[4,5-^{3}H]leucine (spec. act. 55 Ci/mmol) in Eagle's medium without serum. They were washed 3 times with cold PBS, twice with cold 10% (v/v) perchloric acid–2% (w/v) phosphotungstic acid and twice with ethanol cooled to −20°C. The air-dried monolayers were dissolved in hot (80°C) 1 N NaOH (0.5 ml) and aliquots were taken for protein determination [9] and for measurement of ^{3}H radioactivity incorporated into acid precipitable protein. For the latter, aliquots (0.2 ml) were mixed with 10 ml colloidal silica gel scintillation

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fluid [10] and the solution counted in a liquid scintillation spectrometer.

In some experiments the cell monolayers were first treated in PBS (0.5 ml) for 30 min at 37°C with *Vibrio cholera* neuraminidase (Behringwerke-Hoechst, London) at 50 U/ml final conc. [11]. The cell monolayers were then rinsed twice with PBS before addition of toxins and measurement of protein synthesis as above.

To measure binding of ricin and the *Viscum album* lectin to cells, the lectins (~100 μg) were radiolabelled using 250 μCi carrier-free [^{125}I]iodide (Amersham Intl., Bucks) by the iodogen procedure [12]. Labelled lectins were purified first by extensive dialysis against PBS at 2°C followed by affinity chromatography on columns (~5 ml) of Sepharose 6B (Pharmacia, Uppsala). Before use, the Sepharose 6B beads were heated at 50°C for 3 h in 0.2 M HCl to expose additional terminal galactose residues. The radioactive fractions eluted with 0.1 M lactose in each case were pooled and further dialysed extensively against PBS at 2°C. These products were diluted with unlabelled toxins to final spec. act. 10^5–5 × 10^6 cpm/mg protein. ^{125}I-Labelled lectins were added in 0.5 ml PBS to washed, confluent monolayers of cells grown in 24 well Limbro culture vessels. After incubation at 2°C for 1 h the cells were washed 6 times with PBS (each time 1 ml) at 2°C. The cells were dissolved in 1 N NaOH (0.5 ml) and aliquots were taken for determination of protein concentration and for ^{125}I radioactivity in a γ-spectrometer. Binding was estimated as cpm/mg cell protein. To calculate no. binding sites/cell, these data were plotted according to [13].

3. Results and discussion

Hamster fibroblasts (BHK cells) are very sensitive to ricin, as measured by plating assays and by inhibition of total cellular protein synthesis [7,11]. Here, we show that the concentration required to inhibit protein synthesis by 50% of control (LD_{50}) is ~80 ng/ml (table 1). Three cell lines Ric^{R}14, 19 and 21 selected [7] for resistance to ricin are ≥150-times as resistant as the parental BHK cells (fig.1, table 1).

The ricin-resistant BHK cell variants Ric^{R}14, 19 and 21 are also cross-resistant to *Viscum album* lectin (fig.1). This lectin is considerably less inhibitory than ricin on BHK cells, requiring prolonged incubation and at 2 μg/ml to inhibit protein synthesis by 50% of the controls (table 1). By contrast, the ricin-resistant cell lines are as sensitive to the very high toxicity of modeccin as the parental BHK cells (table 1). Therefore, *Viscum album* lectin and ricin must share common steps in exerting their cytotoxicity. By contrast, modeccin appears to act by a pathway independent of the 2 other galactose-binding lectins. This difference is probably expressed at the level of the surface car-

Table 1
Sensitivity of protein synthesis in various cells to the toxicity of galactose-binding lectins

	LD_{50} (ng/ml)				
	BHK	Ric^{R}14[a]	L	BL8L	Ric^{R}14 +neuraminidase
Ricin	80	12000	50	10	40
Modeccin	4	6	7	0.4	ND
V. album lectin	2000	35000	500	7	235

[a] Similar results were obtained for Ric^{R}19 and 21 n.d., not determined

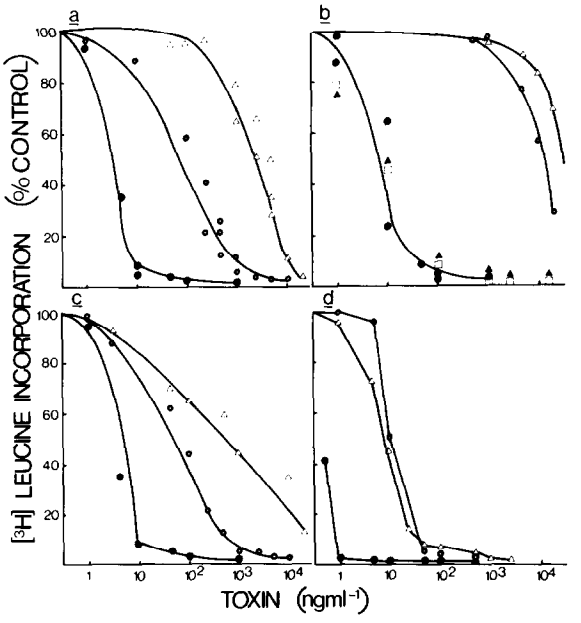

Fig.1. Sensitivity of various cells to 3 galactose-binding lectins. Confluent monolayers were incubated with increasing concentrations of ricin (⊙), modeccin (●) or *Viscum album* lectin (△) at 37°C for 3 h (⊙,●) or 18 h (△). Protein synthesis was then measured as in section 2: (a) BHK cells; (b) Ric^{R}14 cells; (c) L-cells; (d) BL8L cells. In (b) the effect of modeccin on Ric^{R}19 (▲) or Ric^{R}21 (□) cells is also shown.

bohydrate receptors involved in the initial interactions with the cells. Our data agree with [14] where sensitivity of ricin-resistant HeLa cells to modeccin and vice versa was reported.

To study further the specificity of action of the 3 lectins on cells, we used 2 other cell lines of mouse and rat origin. Mouse L-fibroblasts are sensitive to ricin, *Viscum album* lectin and modeccin in relative degree very similar to BHK cells (fig.1, table 1). By contrast, the rat hepatocyte cell line BL8L is significantly more sensitive to ricin and modeccin and dramatically more sensitive to *Viscum album* lectin than either hamster or mouse fibroblasts. LD_{50} of 7 ng/ml for the latter lectin is very similar to that determined using similar techniques for BL8L cells [3].

Results in [15,16] for ricin cytotoxicity suggested that the toxic effect of lectins such as ricin is determined to an important degree by the extent of binding of the toxin to the cell surface. Our own data with many ricin-resistant BHK cell lines is consistent with this view, although exceptions do occur. Thus, the Ric^R19 cell line showing great resistance to ricin (fig.1, table 1) nevertheless binds as much ricin as do parental BHK cells; the reason for their extreme resistance to ricin is unknown. BL8L cells that are most sensitive to the *Viscum album* lectin do bind considerably more of the lectin than either BHK or L-fibroblasts (table 2). The ricin-resistant cell line binds only ~10% of this lectin compared with parental BHK cells. Thus, there appears in this case to be a general quantitative relationship between the number of surface receptors for *Viscum album* lectin and the sensitivity of the cell lines to lectin cytotoxicity. However, the relationship is not impressive: a <10-fold increase in binding of mistletoe lectin results in an increased sensitivity of 70–300-fold. Furthermore, a positive correlation was not found for ricin (fig.2, table 2).

Table 2
Binding sites for ricin and *Viscum album* lectin on various cells

	No. sites/cell X 10^{-6}			
	BHK	Ric^R14	L	BL8L
Ricin	6	0.4	2.3	1.7
V. album lectin	8	0.9	3.4	40

Binding data were analysed as in [13] and separate determination of the cell numbers/mg protein

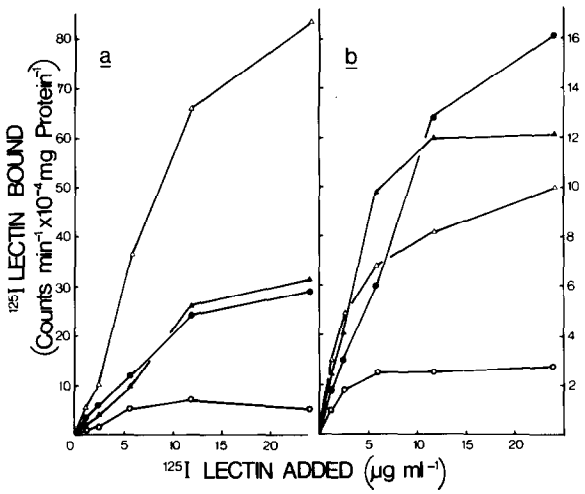

Fig.2. Binding of ^{125}I-labelled lectins to cells. Radio-iodinated lectins were added in increasing concentrations to confluent cell monolayers. After incubation at 2°C for 1 h the radioactivity associated with the washed monolayers was measured as in section 2: BHK cells (●); Ric^R14 cells (○); L cells (▲); BL8L cells (△). (a) *Viscum album* lectin; (b) ricin.

Although Ric^R14 cells bind ~10-fold less ricin than BHK cells or L-cells [7], ricin binding to BL8L cells is also significantly reduced, although these cells are more sensitive to ricin than the fibroblastic cell lines. Therefore, the expression of glycoproteins able to bind ricin and related toxic lectins is not sufficient to determine the sensitivity of those cells to lectin toxi-

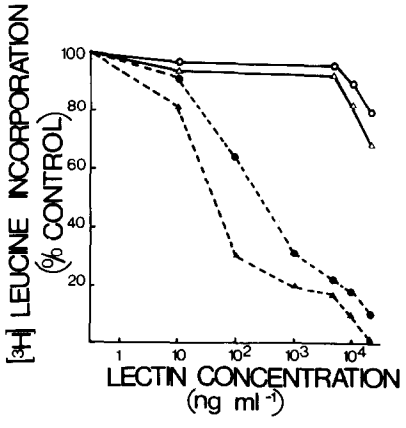

Fig.3. Effect of neuraminidase on the sensitivity of Ric^R14 cells towards galactose-binding lectins. Confluent cell monolayers were treated with neuraminidase (●,▲) before addition of the lectins and measurement of protein synthesis as in fig.1: (○,△) control cell monolayers; ricin (○,●); *Viscum album* lectin (△,▲).

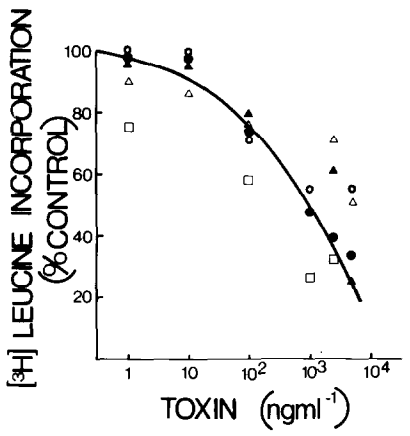

Fig.4. Effect of diphtheria toxin on cells. Confluent monolayers were treated at 37°C for 3 h with diphtheria toxin at the final concentrations shown before addition of [^{3}H]leucine and further incubation for 1 h. BHK cells (○); Ric^{R}14 cells (●); Ric^{R}19 cells (▲); Ric^{R}21 cells (□); BL8L cells (△).

city. Presumably, the cell surface is equipped with specific glycoproteins especially well-adapted to facilitate subsequent steps in toxin action, i.e., in the uptake of the toxins into the cytoplasm, and these may represent different proportions of the total lectin binding glycoproteins on different cells.

The similarity of productive surface receptors for ricin and *Viscum album* lectin implied by the cross-resistance or ricin-resistant cells to the latter is consistent with the effect of neuraminidase on the sensitivity of cells to lectin cytotoxicity (fig.3). Treatment of ricin-resistant BHK cell lines with neuraminidase reveals new binding sites that render the cells sensitive to ricin toxicity [11]. A very similar effect is shown for *Viscum album* lectin using Ric^{R}14 cells (fig.3). The sensitivity of neuraminidase-treated cells is increased by ~150-fold (table 1).

Diptheria toxin recognises glycoproteins present on the surface of susceptible cells [6], and circumstantial evidence [17] points to a role of the mannose-containing carbohydrate moieties of these glycoproteins in toxin binding. The fact that all of the cell lines tested here were almost equally sensitive to the toxin (fig.4) does imply a common determinant, perhaps carbohydrate, present on the surface of hamster, mouse and rat cells of diverse tissue origin. Clearly, the glycosylation defects present in Ric^{R}14 cells have no effect on the sensitivity of the cells to diphtheria toxin, excluding the possibility that this toxin shares surface receptors with ricin and *Viscum album* lectin. The possibility that modeccin and diphtheria toxin share common receptors cannot, however, be ruled out.

These results show that the toxic effects of various lectins are not related in a simple way to the relative numbers of receptors present at the surface of sensitive cells. They also point out the heterogeneity of galactose-containing glycoproteins at the cell surface. The enzyme deficient in Ric^{R}14 cells is a specific *N*-acetylglucosaminyltransferase [18] that is crucial in the processing and elongation of oligomannosidic N-glycans into complex forms containing galactose. The predominant N-glycans of the glycoproteins of parental BHK cells are of the bi-, tri- and tetra-antennary type [19] that have been shown to bind ricin and presumably *Viscum album* lectin, particularly after removal of some terminal sialic acid residues to expose galactose end groups [20]. Since a small but significant amount of activity of the specific glycosyltransferase is detected [18] in Ric^{R}14 cells and the cells do bind a significant amount of ricin and *Viscum album* lectin (fig.2) it may be that sufficient complex N-glycans are still produced to bind the amount of modeccin required to exert its extreme cytotoxicity. Alternatively, the modeccin receptors may be galactose-containing sequences present in other carbohydrate groups, for example in O-glycans, the synthesis of which appears to be undisturbed in Ric^{R}14 cells (D. Stojanovic, R. C. H., unpublished).

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